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Pathogenesis of novel reassortant avian influenza virus A (H5N8) Isolates in the ferret ☆

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ABSTRACT

Outbreaks of avian influenza virus H5N8 first occurred in 2014, and spread to poultry farms in Korea. Although there was no report of human infection by this subtype, it has the potential to threaten human public health. Therefore, we evaluated the pathogenesis of H5N8 viruses in ferrets. Two representative Korean H5N8 strains did not induce mortality and significant respiratory signs after an intranasal challenge in ferrets. However, ferrets intratracheally infected with A/broiler duck/Korea/Buan2/2014 virus showed dose-dependent mortality. Although the Korean H5N8 strains were classified as the HPAI virus, possessing multiple basic amino acids in the cleavage site of the hemagglutinin sequence, they did not produce pathogenesis in ferrets challenged intranasally, similar to the natural infection route. These results could be useful for public health by providing the pathogenic characterization of H5N8 viruses.

Influenza viruses have a broad host range including birds and mammals. Highly pathogenic avian influenza virus (HPAI) is very contagious and induces multiorgan systemic disease in poultry leading to mortality (Swayne and Suarez, 2000). According to the World Health Organization (WHO), human infections by the H5N1 strain of HPAI have increased and show a high mortality of up to 59% (WHO/GIP, 2014). Most human cases were attributed to direct contact with H5N1 from infected poultry. Although human-to-human transmission has been very rarely reported, the H5 varieties of HPAI are still considered to be the most likely candidates for human pandemics (Shinya et al., 2006). In Korea, outbreaks of HPAI H5N1 have been reported four times in poultry farms since 2003 and have caused great concern for public health and the economy (Kim Hye-Ryoung et al., 2012). However, to date, there has been no human case of avian influenza including H5N1 in Korea.

Some of the H5 and H7 influenza viruses have multiple basic amino acids in the hemagglutinin (HA) cleavage site, which is a genetic feature of HPAI (Senne et al., 1996). Most of the HPAI strains produced considerable mortality in chickens with an avian receptor (α2,3-linked sialic acid) distribution, predominantly in the respiratory tract (Salomon et al., 2006). Ferrets and mice are widely used to characterize the pathology of influenza viruses (Belser et al., 2007). In particular, ferrets are known to be susceptible to influenza virus infection and show fever and clinical respiratory signs such as nasal discharge, sneezing, and coughing, like humans (Maher and Joanne, 2004). Therefore, the ferret is the most critical animal model to evaluate the pathogenicity of newly emerging influenza viruses. The recent H5N1 strain has produced high mortality in human because most individuals do not have any T cell memory against it, unlike seasonal influenza viruses. Furthermore, the H5 varieties of HPAI are antigenically evolved to interact with the human (α2,6-linked sialic acid) receptors without the need for an antigenic shift in an intermediate host (Herfst et al., 2012).

Amid general concern about potential pandemics caused by H5N1, a novel reassortant H5N8 HPAI infection occurred in a duck breeding farm in Gochang province, in the southern part in Korea, and spread throughout the country in 2014 (Lee et al., 2014). The first H5N8 outbreak was reported in Ireland in 1983 (Swayne, 2008) where turkeys were the most susceptible hosts to H5N8 infection but ducks could not produce the virus. Following an H5N8 outbreak in China in 2010, it was reported that novel H5N8 viruses arose by continuous genetic reassortment and evolution (Zhao et al., 2013).

To evaluate the risk of human infection by H5N8, it is important to understand the characteristics of the virus and prepare con trol measures against the next round of pandemics. Therefore, we evaluated pathogenesis in ferret model of the novel reassortant HPAI H5N8 viruses isolated from poultry in Korea in 2014.

☆Two H5N8 Korean isolates had low pathogenesis in intranasally challenged ferrets.

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Results

Pathogenicity of Korean H5N8 isolates in ferrets

When ferrets were challenged intranasally with H5N8 viruses, they did not show any mortality in either group. Half of ferrets among 8 numbers of Buan2-infected started to show mild respiratory signs such as sneezing and clear nasal discharges after 3 dpi, whereas the Gochang1-infected ferrets showed no clinical respiratory signs. The highest body weight losses were similar in both Buan2-infected ferrets (8.3% at 10 dpi.) and Gochang1-infected ferrets (7.5% at 10 dpi.). Fever was higher in the Buan2-infected ferrets (1.6 °C at 5 dpi.) than in the Gochang1-infected ones (0.7 °C at 5 dpi.) (Table 1). Buan2 virus infected ferrets maintained fever for 7days and Gochang1 virus infected one returned to normal temperature at 6 dpi. Viruses were shed in nasal washes for 5 dpi in Buan2-infected ferrets but only at 1 dpi in Gochang1-infected ferrets (Table 2). Viruses were detected in the nasal turbinate of Buan2-infected ferrets for 5 days with a decreased viral titer from 10^3 EID_{50}/ml at 1 dpi to $10^{2.5}$ EID_{50}/ml at 5 dpi, suggesting that the virus did not get through the lower respiratory tract to reach the lung. In contrast, viruses from the nasal turbinate in Gochang1-infected ferrets were only secreted until 3 dpi. However, Gochang1 was detected only up to 1 dpi in the nasal washes (Table 2). These viruses were not detected in other organs including respiratory ones. To induce more pathogenesis in ferrets, we applied a more aggressive intratracheal challenge with the two types of viruses. There was no mortality and significant clinical signs in ferrets intratracheally challenged with 10^7 $TCID_{50}$/ml of Gochang1, while ferrets similarly infected with Buan2 showed 100% mortality at a dose of 10^7 $TCID_{50}$/ml and 50% mortality at doses of both 10^6 $TCID_{50}$/ml and 10^5 $TCID_{50}$/ml. Most of the ferrets challenged with the Buan2 virus showed diarrhea and severe body weight loss before death. In the infected lung tissues, Buan2 was detected until 7 dpi but Gochang1 had cleared after 5 dpi. An intranasal challenge had no impact on lungs for either viral strain, whereas an intratracheal challenge with Buan2 induced swelling and edema finally resulting in severe pneumonia. The ferrets challenged with Gochang1 via the trachea showed mild signs of pneumonia until 5 dpi but recovered after that (data not shown) (Table 3).

In terms of histopathology, the ferrets infected intranasally showed mild inflammation in alveoli up to 5 dpi for Buan2 and up to 3 dpi for Gochang1. In the alveoli of ferrets challenged intratracheally with Buan2, severe inflammation was induced after 3 dpi and fibrous pneumonia was observed at 5 and 7 dpi. Intermediate inflammation was observed at 5 dpi in the alveoli of Gochang1-infected ferrets but the alveoli recovered at 7 dpi with only mild inflammation (Fig. 1).

Immunohistochemistry results were consistent with the viral titers in tissues. Only the intratracheally challenged ferrets showed immunostaining for viral antigens in their alveoli: up to 7 dpi for Buan2 and 5 dpi for Gochang1 (Fig. 2).

Both intranasal and intratracheal challenges with these viruses induced weak HI titers against each strain and cross-reactivity between Buan2 and Gochang1 viruses was confirmed (Table 4).

Discussion

Outbreaks of the HPAI H5N8 in poultry farms were reported in Ireland in 1983 (Swayne, 2008) and in China throughout 2009 and 2010 (Zhao et al., 2013). Pathogenesis of H5N8 has been demonstrated only for birds such as chickens, turkeys, and ducks, but no human infection has been reported. The Korean H5N8 viruses isolated in 2014 were classified as highly pathogenic, possessing multiple basic amino acids at the cleavage site of the HA sequence: Gochang1 has LREKRRKR/GLF motifs and Buan2 has LRERRRKR/GLF motif (Lee et al., 2014) which showed L at position 9 with a deletion at position 4 of the HA cleavage site (Kang, 2015). Kang et al reported that Korean isolated H5N8 viruses were moderately pathogenic in wild mallard ducks and did not cause severe illness or death. However viral replication and shedding were greater in H5N8 infected mallards than in H5N1 infected mallards (Kang, 2015).

Table 1
Clinical signs of Korean H5 isolates-infected ferrets.

Virus	Subtype	Body weight	Temperature	Survival[b]	Respiratory[b]	Lethargy
		Loss (%)	Rise (°C)			
A/broiler duck/Korea/Buan2/2014	H5N8	8.3	1.6	8/8	4/8	Not severe
A/breeder duck/Korea/Gochang1/2014		7.5	0.7	8/8	0/8	None
A/chicken/Korea/ES/2003[a]	H5N1	5	NA	3/3	0/3	Not severe
A/Vietnam/1204/2004[a]		22	NA	1/3	2/3	Severe

NA – not available.
[a] This experiment was conducted by CDC, USA (Kwon et al., 2014).
[b] No. of animal/total.

Table 2
Viral titration of nasal washes in Korean H5N8 isolates-infected ferrets by challenge routes.

Virus	Route	Viral titer (log EID_{50}/ml)[a]				
		1 dpi	3 dpi	5 dpi	7 dpi	9 dpi
A/broiler duck/Korea/Buan2/2014	IN	3 ± 0.5	2.5 ± 0.0	2.5 ± 0.5	ND	ND
	IT	ND	ND	ND	ND	ND
A/breeder duck/Korea/Gochang1/2014	IN	2.5	ND	ND	ND	ND
	IT	ND	ND	ND	ND	ND
A/chicken/Korea/ES/2003[b]	IN	5.2	4.4	3.6	ND	NA
A/Vietnam/1204/2004[b]		5.4	5.2	4.2	2.8	NA

dpi – days post infection, IN – intranasal, IT – intratracheal, ND – not detected, NA – not available.
[a] Data are represented as mean ± SD.
[b] This experiment was conducted by CDC, USA (Kwon et al., 2014).

Table 3
Viral titration from organs of Korean H5 isolates-infected ferrets with by challenge routes.

Virus	Route	Viral titer (log EID_{50}/ml)[a]								
		3 dpi			5 dpi			7 dpi		
		Nasal turbinate	Trachea	Lung	Nasal turbinate	Trachea	Lung	Nasal turbinate	Trachea	Lung
A/broiler duck/Korea/Buan2/2014	IN	3.0 ± 0.2	ND	ND	2.0 ± 0.4	ND	ND	ND	ND	ND
	IT	ND	2.0 ± 0.3	6.0 ± 0.5	3.5 ± 0.5	2.5 ± 0.6	3.5 ± 0.5	ND	ND	3.0 ± 1.0
A/breeder duck/Korea/Gochang1/2014	IN	2.0 ± 0.3	ND	ND	ND	ND	ND	ND	ND	ND
	IT	ND	ND	3.0 ± 0.3	ND	2.0 ± 0.4	3.0 ± 0.6	ND	ND	ND
A/chicken/Korea/GJ/2008[b]	IN	NA	NA	3.1	NA	NA	NA	NA	NA	NA
A/Vietnam/1204/2004[b]		NA	NA	3.8	NA	NA	NA	NA	NA	NA

dpi – days post infection, IN – intranasal, IT – intratracheal, ND – not detected, NA – not available.

[a] Data are represented as mean ± SD.

[b] This experiment was conducted by CDC, USA (Kwon et al., 2014).

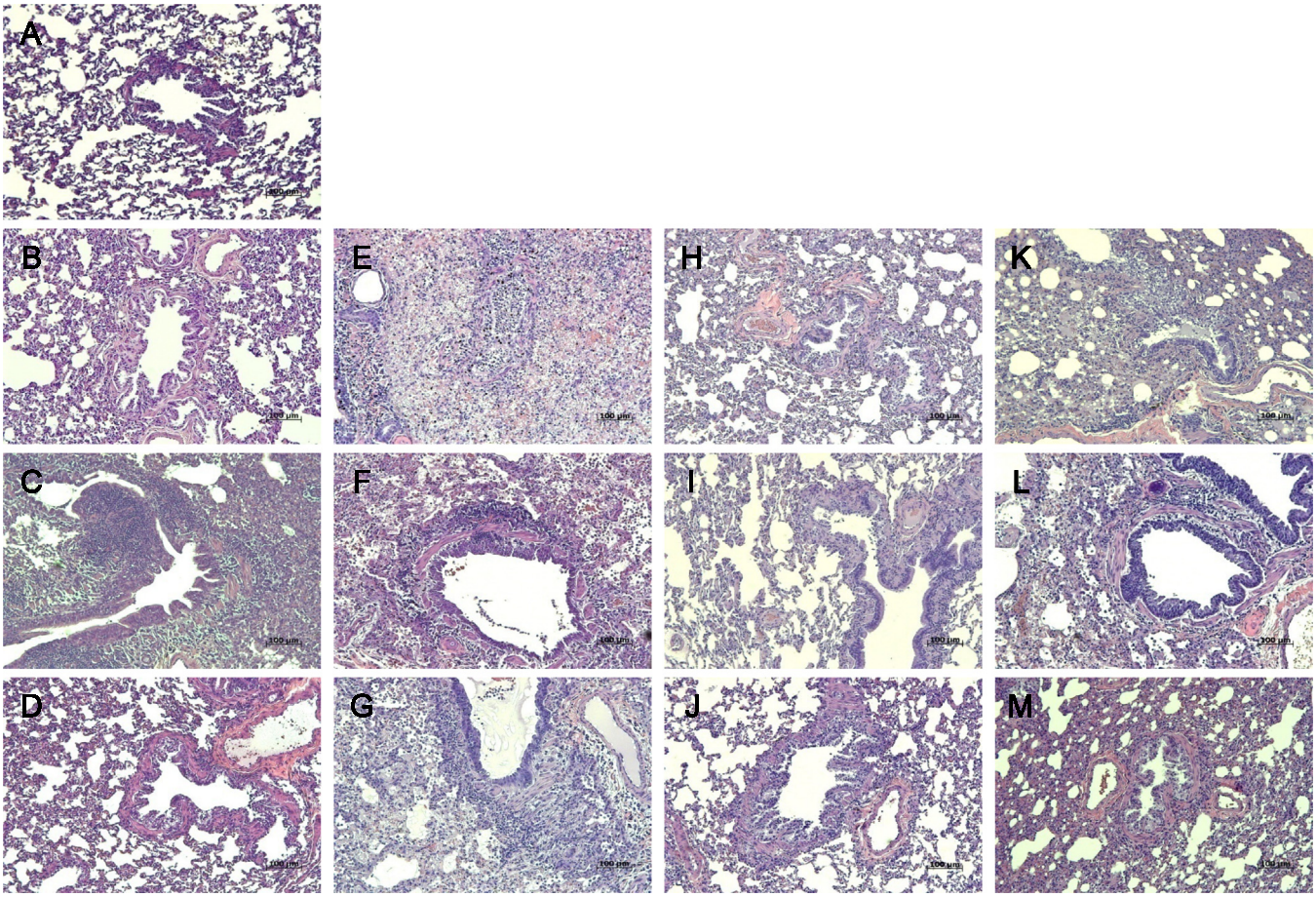

Fig. 1. Hematoxylin and eosin staining of H5N8-infected ferret lungs. (A) PBS mock-infected tissues. (B–G) Effects of Buan2 with lungs collected following two routes of infection. Intranasal infection at (B) 3, (C) 5, and (D) 7 dpi; intratracheal infection at (E) 3, (F) 5, and (G) 7 dpi. (H–M) Effects of Gochang1 with lungs collected following two routes of infection. Intranasal infection at (H) 3, (I) 5, and (J) 7 dpi; intratracheal infection at (K) 3, (L) 5, and (M) 7 dpi. All micrographs were taken at × 100 magnification.

Although there has been no case of human being infected in Korea from the beginning of the H5N8 outbreak, there is still the need to evaluate the possibility of human infections. Therefore, we investigated the pathogenicity of two H5N8 viruses, Gochang1 and Buan2, in ferret. The pathogenesis of influenza viruses varies between hosts with numerous factors involved. For example, the HPAI H5N1 was lethal against chickens but did not kill ducks, whereas it showed low to high virulence in ferrets. Ferrets have an influenza virus receptor distribution similar to humans (Herfst et al., 2012) and show similar clinical signs after infection (Reuman et al., 1989). Therefore, the ferret is considered an appropriate animal model for assessing the human risk from influenza viruses. An intranasal challenge is a general method for estimating pathogenesis in ferrets as it leads the virus to infect the URT, similar to the route of natural infection.

In this study, when ferrets were intranasally administered with the Buan2 or Gochang1 viruses, they did not show any significant clinical signs and viruses were not detected in the lower respiratory tract. Viral titers in major organs including the lungs were not detected and immunostaining for the H5N8 viruses showed negative in intranasally inoculated ferrets. Virus-infected lungs showed mild inflammation in the alveoli at 5 dpi and did not show any viral antigen.

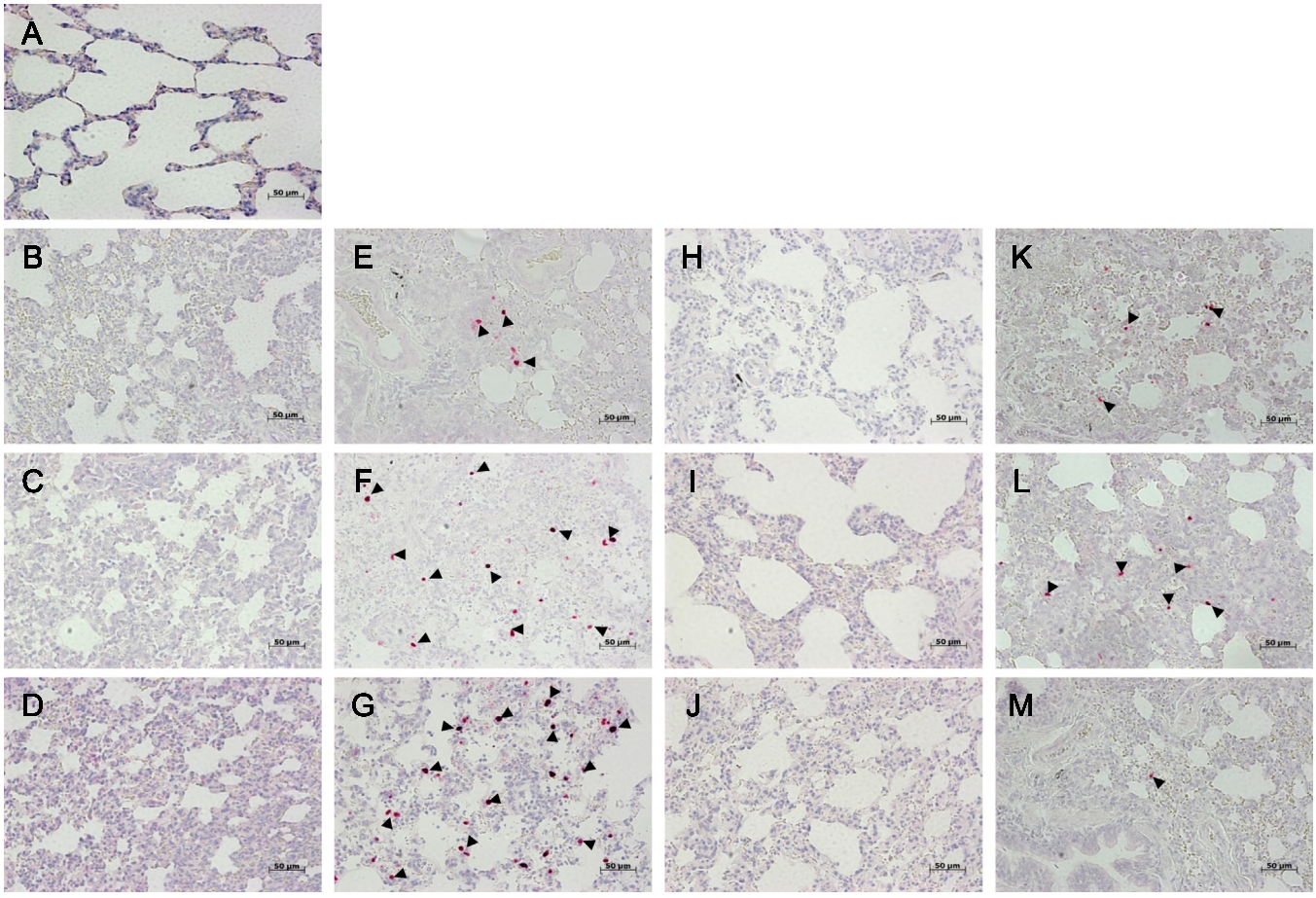

Fig. 2. Immunohistochemistry of viral antigen in lung tissues from ferrets infected with H5N8 isolates. (A) PBS mock-infected control tissues. (B–G) Effects of Buan2 with lungs collected following two routes of infection. Intranasal infection at (B) 3, (C) 5, and (D) 7 dpi; intratracheal infection at (E) 3, (F) 5, and (G) 7 dpi. (H–M) Effects of Gochang1 with lungs collected following two routes of infection. Intranasal infection at (H) 3, (I) 5, and (J) 7 dpi; intratracheal infection at (K) 3, (L) 5, and (M) 7 dpi. All micrographs were taken at × 200 magnification.

Table 4
Heamagglutination inhibition test with sera of Korean H5N8s infected ferrets.

Antiserum against	Route	Reciprocal HI titer[a]	
		Buan2	Gochang1
A/broiler duck/Korea/Buan2/2014	IN	80	40
	IT	160	80
A/breeder duck/Korea/Gochang1/2014	IN	40	80
	IT	40	80

IN – intranasal, IT – intratracheal.
[a] Data are represented as mean.

According to the study of Rogier et al., Intranasal inoculation in ferrets resulted in primary disease of the CNS rather than of the respiratory tract (Bodewes et al., 2011). However, in our study H5N8s infected ferrets did not show significant clinical sign and limited viral isolation only in upper respiratory tract (URT) without CNS infection. This conflicting result with other HPAI study in ferrets was attributed to genetic characteristic of H5N8s featuring avian receptor preference in HA and insufficient mutation of polymerase genes for acquisition mammalian pathogenesis.

Korean isolated H5N8 had a genetic characteristic in HA retaining α2,3-linked sialic acids (α2,3-SAs) affinity (Kim et al., 2014). Genetically, amino acid substitution in HA related in increasing virus binding to α2,6-linked sialic acids (α2,6-SAs) was not sufficient (data not shown). Because α2,6-SAs mainly exists in URT of ferrets, the discrepancy between avian receptor (α2,3-SAs) preferred H5N8s and abundant human receptor (α2,6-SAs) in URT of ferret was primary reason to restrict virus isolation in ferret. Therefore we designed more aggressive intratracheal challenge method to exclude the constraints and could induce pulmonary disease in Buan2 virus infected ferrets. Furthermore, E^{627} in PB2 of Korean isolated two H5N8s played critical role in host range restriction and decreased virulence in mammalians (Salomon et al., 2006). In addition, innate immunity in mucosal of URT assist to obstruct the entry of influenza virus in lung of ferret (Maines et al., 2012). We considered that those factors might affect low pathogenesis and limited viral isolation in URT of ferrets without systemic infection, even though Korean isolated two H5N8s which belong to HPAI possessing multi-basic cleavage site (MBCS) on the hemagglutinin. Some HPAI case was reported that systemic infection was not detected in mammalians (Maines et al., 2005).

The prolong and brief shedding periods of Buan2 and Gochang1 viruses respectively could be explained by differences in adaptation in mammalian cell between two viruses. Even though they shared same genetic property in Polymerase genes including PB2 I^{63}, D^{256}, Q^{591}, E^{627} and D^{701}, PB1 K^{207}, Y^{436} and T^{677}, PB1-F N^{266} and PA A^{515} related mammalian host adaptation (Centers for Disease Control and Prevention (CDC), 2012), the growth kinetics and plaque size of Buan2 virus were 10^2 fold higher ($TCID_{50}$/ml) and larger than Gochang1 virus in MDCK cells (data not shown). Yong-Gang Li

et al. suggested, H5N1 replicated slowly in MDCK cells and producing small plaque induced low pathogenesis in ferrets (Li et al., 2010).

Compared with a few representative H5N1 viruses previously isolated in Korea, the pathogenesis of these H5N8 isolates in ferrets showed similar patterns, with low pathogenicity to ferret. However, when ferrets were infected with the Buan2 virus via the trachea, they exhibited a more pathogenic response in that viruses were detected in the lungs but not in other organs. Although this aggressive challenge produced mortality in the ferrets, it is the less representative of natural infection due to the rare chance administered to lower respiratory tract directly with excessive amount of virus in nature. Similarly, H7N9, known as a low pathogenic avian influenza virus, could cause death in ferrets using an intratracheal challenge (Kreijtz et al., 2013). Accordingly, we need further studies to investigate the suitable dose of influenza viruses for intratracheal challenge in ferrets to evaluate pathogenesis. Taken together, our results and the known genetic characteristics of Korean H5N8 isolates (Lee et al., 2014) suggest that there might be little risk of human infection by these viruses. However, our ferret experiment used a limited number of animals and it might not reflect individual human traits such as age and immune status, it is still needed to monitor and evaluate the risk of a human infection.

Conclusions

Even though we found that these two H5N8 Korean isolates had low pathogenesis against ferrets, we cannot exclude the possibility of human infection. Therefore, we should consistently monitor and assess the characteristics of H5N8 viruses by analyzing their genetic traits and conducting further animal experiments.

Methods

Ethics statement

All experimental animal studies were performed in the animal biosafety level 3 (ABL3) facilities at the Korea Centers for Disease Control and Prevention (KCDC), with approval (KCDC-116-14-2A) of the animal usage and safety protocols by the Institutional Animal Care and Use Committee of KCDC.

Virus preparation

Two Korean H5N8 isolates were kindly provided by the Avian Disease Division of the Animal and Plant Quarantine Agency (QIA) in South Korea. The A/breeder duck/Korea/Gochang1/2014 virus (Gochang1) was the first isolated and the A/broiler duck/Korea/Buan2/2014 virus (Buan2) was one of the epidemic strains circulated in Korea. The viruses were grown in the allantoic fluid of 10-day-old embryonated chicken eggs for 36 h at 37 °C. Virus stocks were aliquoted and stored at −70 °C until use. Viral concentrations were checked using the 50% tissue culture infectious dose ($TCID_{50}$/ml) in Madin–Darby canine kidney (MDCK) cells based on the method of Reed and Muench (Reed and Muench, 1938).

Virus infection in ferrets

To evaluate the pathogenesis of HPAI H5N8 *in vivo*, we used 6-month-old female ferrets (Woojung BSC, Inc., Suwon, South Korea). The ferrets were confirmed to be serologically negative for currently circulating seasonal human influenza A/California/07/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Brisbane/60/2008, and B/Massachusetts/2/2012 by hemagglutination inhibition (HI) assays before the study. Ferrets were anesthetized using an intramuscular injection of a cocktail of ketamine (24 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg) and infected intranasally (1 ml of 10^7 $TCID_{50}$/ml) and intratracheally (1 ml of 10^4–10^7 $TCID_{50}$/ml) with H5N8 influenza viruses in PBS.

Pathogenicity in ferrets

Nine ferrets were allocated randomly for each virus, and baseline body weights and body temperature were measured before inoculation. One ferret was inoculated with sterile PBS as a mock control. Two infected ferrets were each euthanized at 3, 5, and 7 dpi and tissue specimens (lung, trachea, nasal turbinate, spleen, kidney, liver, heart, intestine, and brain) were collected for measuring viral titers. Five lobes of lung were collected and pulled for viral titration and right lung (cranial, medial and caudal lobe) was used for histopathology (Vidana et al., 2014). Ferrets were monitored two times a day (9:00am and 18:00pm) for clinical signs, body weights, and body temperature for 14 days. Activity and clinical scores were estimated as described previously (Reuman et al., 1989). Body temperature was measured rectally with digital thermometer. Nasal washes were collected every other day for 9 dpi to check for viral shedding. To collect nasal wash samples, ferrets were sedated with cocktail of ketamine (24 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg), and 1 ml of sterile PBS containing 1% bovine serum albumin and penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml) was injected into each nostril and collected in a petri dish when expelled by the ferret (Fang et al., 2010).

Viral titration in chicken embryonated eggs

Tissues were homogenized to make a 10% (w/v) suspension using a Qiagen TissueLyser II (Qiagen, Valencia, CA, USA). The amount of infectious virus in tissue homogenates and nasal washes was determined by inoculating the serially diluted sample to chicken embryonated eggs.

Hemagglutination inhibition assay

Prechallenge sera were collected before infection and postchallenge sera were collected at 14 dpi from ferrets. Specific antibodies against H5N8 were evaluated by HI assays using 0.5% turkey red blood cells following the standard WHO protocol (Webster et al., 2002).

Histopathology

Histopathology of animal tissues was performed as described (Kwon et al., 2014). In short, after fixation in 10% neutral-buffered formalin, tissues were processed routinely and embedded in paraffin wax. The wax blocks were cut into 5-μm-thick sections and stained with hematoxylin and eosin for histopathology. For evaluating influenza viral antigen expression, tissues were processed for immunohistochemical staining with anti-influenza A specific antibodies. A monoclonal antibody (Novus Biologicals, Littleton, CO, USA) against the nucleoprotein of influenza A virus was applied for the ferret tissues. Then, the sections were treated with biotinylated anti-mouse (Dako, Glostrup, Denmark). The slides were then treated with alkaline phosphatase-conjugated streptavidin (Merck KGaA, Darmstadt, Germany) and were visualized using a solution of Fast Red alkaline phosphatase substrate (Roche Diagnostics GmbH, Mannheim, Germany). The sections were lightly counterstained with Mayer's hematoxylin and mounted with aqueous mounting medium (Dako, Glostrup, Denmark).

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